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Biologically active metabolites of endophytic *Diaporthe brasiliensis* species from *Cassia fitula* L

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Abstract
Endophytic microorganisms are impending source of bioactive molecules and have shown proved medicinal applications. Modern age techniques involving the use of different organisms living in plant tissues root back in ancient times where leaf extracts were used. Endophyts act as the treasure house of number of biologically active metabolites having proved medicinal values. A crude ethyle acetate extract of the endophytic fungus *Diaporthe brasiliensis*, isolated from the medicinal plant Cassia fistula L, was examined for antibacterial and -amylase inhibitory activities in order to study the biological potential of endophytic fungi. Furthermore, bioactive metabolites have been investigated using analytical technique GC-MS. It was observed that Crude extract of *Diaporthe brasiliensis* showed antibacterial activity at two different concentration 50 and 100µg/l selectively against gram positive bacteria *S. aureus* and *B. subtilis*. Whereas negative result was observed against gram negative bacteria. Further, crude extract of *Diaporthe brasiliensis* was evaluated for α-amylase inhibitory potential which showed antidiabetic potential as like standard molecule acarbose. GC-MS analysis was revealed that crude extract contains various metabolites two molecules 7,9-di-tert-butyl-1-oxaspiro (4,5) deca 6,9-diene and 2,8-dione cholesta-4,6-dien-3-ol, (3beta) whose probable concentration was more than the other molecule.

Keywords: endophytic fungi, cassia fistula. l, antibacterial assay, α-amylase inhibition, 2,8-dione cholesta-4,6-dien-3-ol, (3beta)

Introduction
Endophytes are microorganism with omnipresence inside the plant either they reside in local selected space or roll out the complete plant. Endophytes shows affluent biodiversity and have potential for biosynthesis, but they are remains to investigated to produce a cluster of bioactive metabolites [1]. The specific chemical environment of the plant influences the metabolite profile of cryptic endophytic fungi. The biochemical processes for the synthesis of plant-specific compounds were acquired by the endophytes. Endophytes with this competence to formulate plant-specific metabolic compounds have important role in understanding host endophyte interaction -. Indian labranum (Cassia fistula L) is source of potential medicinally important bioactive compounds gain interest of scientific community for the investigation of endophytic fungal and diversity. The fungal endophytes are considered as future cryptic resource to produce potential bioactive metabolites with great medicinal uses [3]. Few examples of bioactive compounds which are produced from cryptic endophytic fungi is Paclitaxel, which is drug of hope used in cancer treatment. *Taxomyces andreanae*, an endophytic fungus found in Pacific yew trees, is the source of the plant alkaloid in the formulation [4]. Another useful drug in cancer treatment is camptothecin also reported from *Entrophospora infrequens*, an endophytic fungus isolated from *Nothapodytes foetida* small evergreen tree [5]. One more drug which is used in cancer treatment is podophyllotoxin, produces from endophytic fungi *Alternaria sp.* reported from Sabina vulgaris shrubs [6]. Besides the cancer endophytic fungi also well reported with the mixture of compound which shows Antidiabetic, Antimalarial and antiviral activities for instance *Aspergillus awamori* a cryptic endophytic fungus isolated from the *Acacia nilotica* plant has potential to synthesized antidiabetic peptides [7]. Epoxycytochalasin *H* is an endophytic Phomopsis compound obtained from endophytic fungus *Diaporthe miriciae*, exhibiting antimalarial activity. It shows potential biological response against deadliest species of plasmodium (Plasmodium falciparum) that cause Malaria [8]. The antiviral compounds Cytonic acid A ($C_{32}H_{36}O_{10}$) and B ($C_{32}H_{36}O_{10}$) are the potential tridepside inhibitors of hCMV protease obtained from endophytic fungi *Cytonaema spp* [9]. In this research article we are presenting antibacterial activity and amylase inhibitory activity of crude extract of endophytic fungi *Diaporthe brasiliensis* isolated from *Cassia fistula L* (Indian labranum) in our previous studies. The genus Cassia (Fabaceae) comprises about 600 species belongs to tropical and subtropical regions of Southeast Asia, Africa, Northern Australia, and Latin America [10, 11]. *Cassia fistula L* (Indian labranum) are distributed all over the world as an ornamental and

medically important plant [12]. During phytochemicals studies of *Cassia* revealed that it comprises of saponins, alkaloid, flavonoids, steroids, anthraquinones, tannins, triterpenoids and phenolic compounds used as biological important compounds [13].

Materials and Methods

Sample Collection

Fresh and healthy leaves of *Cassia fistula L.* Plants were collected from various locality of Marathwada region of Maharashtra for the isolation of endophytic fungi. The samples were carefully brought in the laboratory placing in sterile plastic bags [14]. The leaves were immediately inoculated after sterilization for endophyte isolation.

Surface sterilization

The leaves were washed properly under running tap water to remove dust particles and waste adherent material. They were sterilized using 70% ethanol up to 30 s, followed by 4% sodium hypochlorite for the time of 2 min. The leaves were rinsed in sterile distilled water three time for 1 min to remove every particle of chemicals. Leaves were then blot dried on sterile Grade 1 Whatman paper.

Isolation of endophytic fungi from explant

The surface-sterilized leaves were cut into small square sections of 5 mm length and inoculated on sterile potato dextrose agar (PDA) medium provided with streptomycin (100 µg/ml) to supress bacterial contamination. The tissue sample for endophyte isolation was the area from the centre of the leaf, close to the midrib, taken from all the explants. After placing explants, the PDA plates were closed with paraffin film and incubated at 28 °C for 5 days or until fungal growth was observed. The effectiveness of sterilization was checked by taking an imprint of every explant on a sterile PDA plate to check if epiphytic microbial growth occurred [15].

Statistical studies

Colonization Frequency (CF %)

To study colonization frequency (CF %) of each isolated endophytic fungal species from the leaf segments was performed by using the formula given by Hata and Futai (1995) [16].

$$CF\% = \frac{\text{Number of segments colonized by an endophytic fungal species}}{\text{Total number of segments}} \times 100$$

Relative percentage occurrence (RPO %) of each group of fungi

Relative percentage occurrence (RPO %) of different isolated group of endophytic fungi was performed by the following formula:

$$RPO\% = \frac{\text{Density of colonization of one fungal group}}{\text{Total density of colonization of all fungal groups}} \times 100$$

Fermentation, extraction, and isolation of Bioactive metabolites

Organic solvent ethyl acetate was used to extract the filter. The growth media and the mycelia were separated from each other by performing filtration process. The remaining liquid portion filtrate was extracted thrice with equal volume of Ethyl acetate solvent. solvent was subjected to liquid - liquid extraction for 3 to 4 times properly. Solid residues were collected by evaporating complete organic solvent from extracts under reduced pressure, solid residue were further used to performed the antibacterial assay.

Screening of endophytic fungi for antibacterial activity

The antibacterial activity of all the isolated and purified endophytic fungi was done and screened against different bacteria using agar well diffusion method. Four bacteria were used in study were procured from National Collection of Industrial Microorganisms located in NCL, Pune.

The bacterial strain were

Gram Positive

1. *Bacillus subtilis* (MTCC No. 8960)
2. *Staphylococcus aureus* (MTCC No. 96)

Gram Negative

1. *Escherichia coli* (MTCC No. 1687)
2. *Pseudomonas aeruginosa* (MTCC No. 3541)

Agar well diffusion test for antimicrobial activity

Among the species showing moderate to significant activity one species were selected for further antimicrobial study. To performed antibacterial assay modified agar well diffusion method was used. A loopful of bacterial culture was inoculated in 5 ml of Muller Hilton broth and incubated at 37^0c for 24 hours. After incubation,

standard inoculum was prepared by inoculating 100 microliter (10^7 CFU/ml) of fresh bacterial culture into soft agar and mixed properly. Plates were prepared using 15 ml of Muller Hilton Agar and inoculated with standardized inoculum. About 6 mm diameter of wells were prepared and filled with two different concentration 50 and 100 microliter respectively with endophytic fungal extracts. Standard antibiotic like Streptomycin and equal quantity of ethyl acetate was used as a positive and negative control, respectively. All the experiments were carried out in triplicates.

α-amylase inhibitory assay

DNSA (Dinitrosalicylic acid) was used as the reagent in all the experiments of -amylase activity that were carried out. Equivalent amounts of purified enzyme with an absorbance of 0.5% at 540 nm were added to the premix, which was produced using 20mM phosphate buffer (pH 7.0) and 150 L of starch (0.25 percent). The assay was run in triplicate. In order to halt the reaction, 500 L Dinitro salicylic acid reagent was used and the reaction tube was placed in a boiling water bath for 5 minutes, following which the absorbance at 540 nm was measured. According to the results of this test, the amount of enzyme required to release 1M maltose/min from starch (substrate) at 37°C was determined to be one -amylase unit. [17]. α-amylase inhibitory assay was carried out using similar method as mentioned above with simple modification, enzyme and inhibitor were added this mixture incubated for 15 min at 37°C than substrate were added. α-amylase inhibitory activity of crude extract of endophytic fungi were tested against human salivary α-amylase.

Gas chromatography-mass spectroscopy (GC–MS) analyses of crude extract of *D. brasiliensis*

Ethyl actate crude extract of *D. brasiliensis* was subjected for analysis of biologically active metabolites using Gas chromatography-mass spectroscopy. The analysis was performed in Sophisticated analytic instrument facility centre, IIT-Bombay, Mumbai. The GC-MS analysis was performed using the Jeol, Accu TOF GCV using EI / CI Source as the source for ionization. The ethyl acetate crude extract was ten time diluted and 1µL was subjected to GC–MS analyses. The NSIT database were used for further identification.

Results and Discussion

Total of 50 segments from *Cassia fistula L* were selected to study the presence of endophytic fungi. Of the 35 isolates of endophytic fungi purified from the leaves segment of *Cassia fistula L* were isolated as summarised in Table 1.

Table 1

1	**Total no of segments used**	**50**
2	Total no of segments yielding endophytes	29
3	Total no of endophytic isolates obtained	35
4	Percentage of Colonization (% CF)	58%

Diversity of Endophytic fungi

Cassia fistula L. leaves harbour diverse species of endophytic fungi there CF% were calculated it is showed that *Diaporthe sp.* and *Colletotrichum sp.* has same CF% value that is 17.25% highest among all the isolates. Relative percentage of occurrence of both species is 10% and 12% respectively. Kuriokose *et al*. 2018 also reported seventeen endophytic fungi which isolated during the study from *cassia fistula L.* during the study on human cervical cancer [18].

Table 2: Analysis of antibacterial screening of crude extract of *D. brasiliensis*

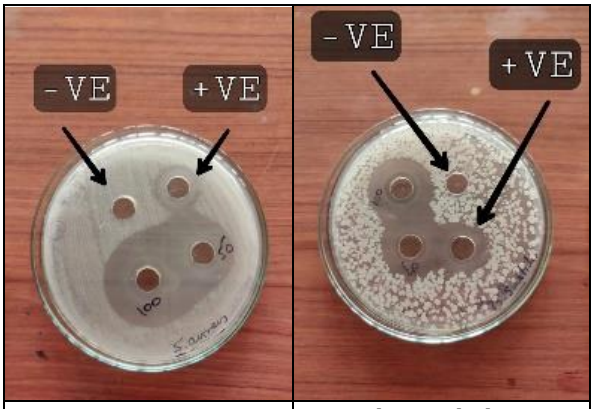
S. No	**Isolates**	***Cassia fistula***		**% RPO**
		NOI	**% CF**	
1	*Chaetomium sp.*	02	6.89%	12
2	*Amesia sp.*	01	3.44%	07
3	*Ovatospora sp.*	02	6.89%	05
4	*Aspergillus sp.*	05	17.24%	10
5	*Penicillium sp.*	04	13.79%	11
6	*Phialemonium sp.*	00	---	05
7	*Colletotrichum sp.*	05	17.25%	12
8	*Crinipellis sp.*	00	---	01
9	*Acrophialophora sp.*	00	---	01
10	*Cribbea sp*	00	---	01
11	*Alternaria sp.*	04	13.79%	08
12	*Diaporthe sp.*	05	17.25%	10
13	*Phyllosticta sp.*	01	3.44%	01
14	*Phomopsis sp.*	02	6.89%	02
15	*Nigrospora sp*	04	13.79%	05
	Total	35		

Table 3: Antimicrobial screening was performed using 4 different bacteria. Result summarized below in table.

S. No	Summary of antibacterial activity	*Cassia fistula L.*
1	Moderate to Significant (at least against one test bacteria)	35
2	Significant antibacterial activity (at least against one test bacteria)	10
3	Some antibacterial activity (Against every one of all four test bacteria)	07
4	Moderate to Significant (Against every one of all four test bacteria)	04

Antimicrobial activity of crude extract of *D. brasiliensis*

Ethyl acetate crude extracts of *D. brasiliensis* was performed strong inhibitory activity. It was observed that Gram positive bacteria (1) *S. aureus* and (2) *B. subtilis* shown strong growth inhibition against the extract. but there was no effective antibacterial activity observed against Gram negative bacteria (1) *E. coli* and (2) *p. aureginosa*.



a. S.aureus | *b. B. subtilis*

Fig 1: Antibacterial activity

A-amylase inhibitory activity of crude extract of *D. brasiliensis*

During the amylase inhibition study, it was found that ethyl acetate crude extract of *D. brasiliensis* was showing 100% inhibitory activity against α-amylase enzyme. Acarbose was used as reference drug.

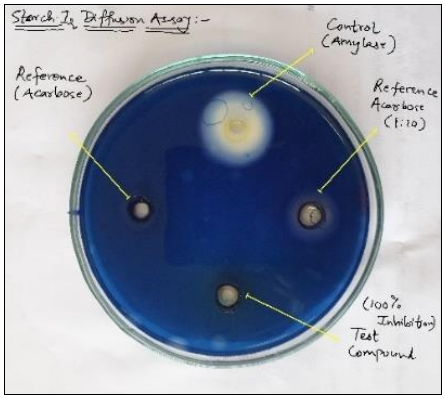


Fig 2: Amylase inhibitory activity

Characterization of metabolites of crude extract of *D. brasiliensis*

The chromatogram of GC analysis revealed the presence of various compounds which was depends on the required retention time, covered peak area, and observed molecular formula all the expected lead and identified molecules are presented in table. The current finding revealed the presence of various compounds and their structures which may be considered as responsible molecules for the antimicrobial activity and α-amylase inhibitory assay present in crude extract of selected endophytic fungi.

Table 4: Mass spectra data of crude extract of *D. brasiliensis*

Peak No	Chemical Name	Chemical Formula	Retention Time(min)	Mol. weight	Prob. (%)
1	Phenol 2,4-bis (1,1-dimethyethyl	$C_{14}H_{22}O$	18.238	206	40.2
2	7,9-di-tert-butyl-1-oxaspiro (4,5) deca 6,9-diene 2,8-dione	$C_{17}H_{24}O_3$	25.703	276	91.0
3	1,2-benzenedicarboxylic acid	$C_{22}H_{34}O_4$	26.450	362	8.63

4	Octadecyl trifluroacetate	$C_{20}H_{37}F_3O2$	28.580	366	4.37
5	Sulfurouse acid, pentyl undecyl ester	$C_{16}H_{24}O_3S$	28.713	306	51.9
6	Eicosane, 2-methyl	$C_{21}H_{44}$	30.006	296	7.20
7	Eicosane, 2-methyl	$C_{21}H_{44}$	33.336	296	14.8
8	Nonadecane, 2-methyl	$C_{20}H_{42}$	36.016	282	4.64
9	Di-n-decylsulfone	$C_{20}H_{42}O_2S$	38.086	346	39.5
10	Heptacosane	$C_{27}H_{56}$	38.492	380	8.13
11	Heptacosane	$C_{27}H_{56}$	38.503	380	17.4
12	cholesta-4,6-dien-3-ol, (3beta)	$C_{27}H_{44}O$	39.816	384	64.6

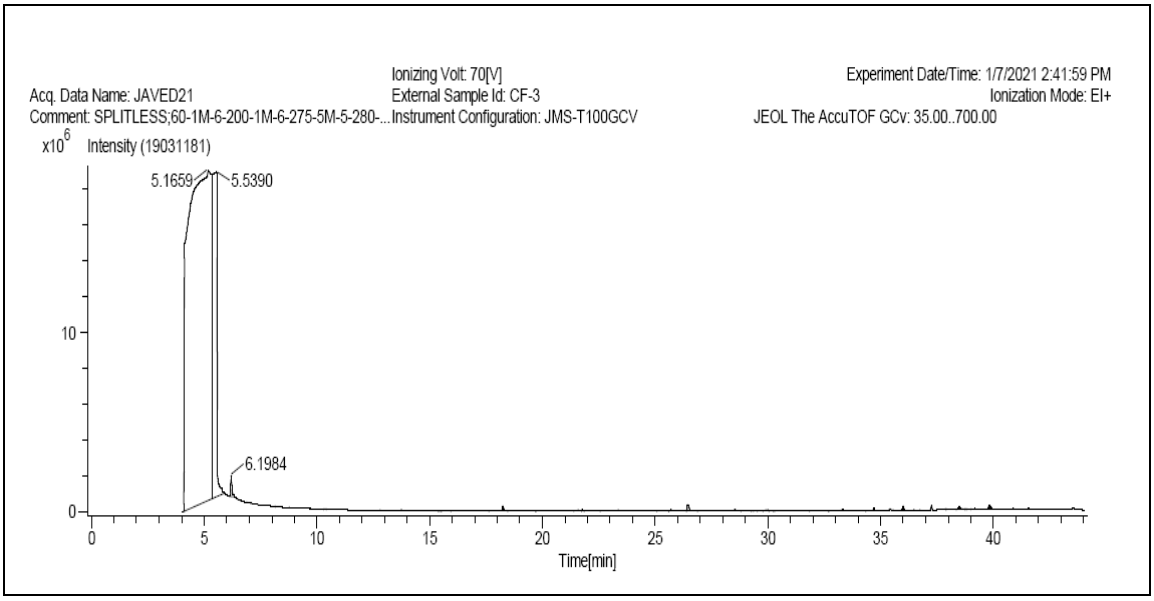


Fig 3: Chromatograms of crude extract of *D. brasiliensis.*

Metabolites derived from Fungi are natural entities have been considered treasure of novel structural molecule with potential antibacterial property. In the present study, antimicrobial activity and α-amylase inhibitory activity were observed for endophytic fungal crude extracts. The crude extracts were used obtained from endophytic fungi *D. brasiliensis* shows considerable and effective antibacterial activity as well as crude extract also performed α-amylase inhibitory activity efficiently. Natural sources like plants were extensively explored for their use in drug discovery but endophytic fungi were remained unexplored and untouched for their contribution in human welfare and eco-friendly medicine development. They are highly diverse and capable to thrive in every stressful environmental condition. Now a days scientist shifted their focus toward the endophytic fungi, and they assumed that they can abstained efficient bioactive metabolites and drug-like molecules [19]. Some *Diaporthe sp.* Previously reported by various researchers with production of bioactive compounds such as diapolic acids which showing potential biological activities like as cytotoxicity and anti-candidal activity. They were also reported to produce some phenolic compounds which has potential antioxidation activity.

Conclusion

The findings indicate that endophytic fungi *D. brasiliensis* can be used to synthesize new natural, nontoxic, and potential bioactive compounds. It can also consider as rich source of bioactive compounds. It is shown that it has potential to produce compounds which have antibacterial and α-amylase inhibitory activity. Efforts are needed to take these compounds forward for drug development. Crude extract of endophytic fungi has numerous bioactive compound which can be separated by using various analytical technique for further investigation.

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